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# **THE ROLE OF PHOTO-ACTIVATED MELANOSOMES AND CELLULAR ANTIOXIDANT DEFENSES IN THE RESPONSE OF RPE CELLS TO LASER RADIATION**

**Final Report  
AFOSR Grant F49620-98-1-0210**

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## **ABSTRACT**

The pigments of the retinal pigment epithelium, i.e. the intracellular granules of melanin, lipofuscin, and melanolipofuscin, have been shown to catalyze free radical activity, especially when illuminated with visible or ultraviolet light. An important issue is whether these reactions are sufficient to produce oxidative damage in the eye. To address this question, the relative photoreactivity of isolated RPE pigment granules towards polyunsaturated fatty acids has been determined, including the dark as well as the light-stimulated reactions. Hydroperoxide derivatives of docosahexaenoic acid were produced by irradiation with short wavelength (<550 nm) visible light when RPE pigments were present. Although melanosomes exhibited the greatest light-induced activity in these reactions, lipofuscin granules induced peroxidation of fatty acids in the dark. In intact, cultured RPE cells, the existence of pigment-mediated photo-reactions was demonstrated with a fluorescent indicator probe of oxidation, 2',7'-dichlorofluorescein, that was photooxidized in probe-labeled cells in a wavelength dependent fashion with peak activity in the 450 to 500 nm region. This behavior resembled the action spectrum for melanin reactivity. These findings support the hypotheses that the RPE pigments in general contribute to photooxidative stress in ocular tissue, and that accumulation of lipofuscin pigment specifically contributes to age-related oxidative stress in the RPE.

## INTRODUCTION AND BACKGROUND

Our previous work [1-10] has explored the photochemical properties of the melanosome and its relation to phototoxicity in the retinal pigment epithelium. Our basic premise has been that photooxidative stress in the eye is directly related to the light-induced reactions of the RPE pigments: melanin, lipofuscin, and melanolipofuscin. All of these pigment granules have been shown to possess photosensitizing properties, i.e. during irradiation with UV and visible wavelengths, they are excited to free radicals, and promote or participate in oxidative reactions.

It has long been known that exposure to visible light excites melanin to a free radical [11,12]. Lipofuscin and melanolipofuscin are pigments associated with age-related changes in the eye [13-15], and are known to produce reactive molecular species (particular oxygen radicals) during irradiation with visible and ultraviolet light [16-18]. Recently, lipofuscin has been shown to cause oxidation of polyunsaturated fatty acids even in the absence of light [19]. Melanin, on the other hand, is conventionally regarded as having a protective role against light damage [20,21], but may also promote light damage under certain conditions because it is a photoinducible free radical [11], produces reactive oxygen radicals when irradiated [22,23], and promotes photochemical reactions with physiological substrates [1,7,24,25].

The other pigment of note in the RPE is lipofuscin. Long considered an "aging" pigment [14,26,27], lipofuscin has at least two UV-induced chromophores, one absorbing maximally at 430 nm and another absorbing at 580 nm [16]. These transient species are reactive and can initiate photooxidative reactions, including the generation of singlet oxygen [16], superoxide anions [28], and lipid hydroperoxides [18]. The decline in melanin content in RPE and choroid with age is often associated with an increase in lipofuscin content [14,26,27], so that an increase in lipofuscin content in RPE cells has been considered a potential cause of increased oxidative stress in the eye [29].

In view of these observations, it was important to characterize the photoactivated, as well as constitutive (dark), reactions of RPE melanin, lipofuscin, and complex (melanolipofuscin) granules, as this information would be necessary to understand the development of photooxidative stress in light or laser exposed pigmented tissue such as the RPE. Several experimental approaches were used to attack this problem, including photochemical studies with isolated RPE pigment granules, and in cultured RPE cells using indicators of oxidative stress. Ancillary studies were directed at determining photophysical properties of the melanosome that related to its ability to interact with laser radiation and undergo either disruption or excitation to a reactive state from which further oxidative reactions could be promoted.

#### *Scope of the project*

In the project reported here, we have concentrated on characterizing the interaction of light-excited melanosomes with the endogenous molecules of the retinal pigment epithelial cell. In order to do this, we isolated human RPE pigment granules, and used them to study photoinduced peroxidation of polyunsaturated fatty acids (PUFA), especially *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA). DHA is an especially appropriate substrate in the context of ocular oxidative damage, because it is the most unsaturated physiological fatty acid, accounts for nearly 25% of the fatty acids in the retina, and is the most common PUFA in photoreceptors [30]. We compared the photoinduced as well as constitutive (dark) reactions of RPE melanin, lipofuscin, and complex (melanolipofuscin) granules in a model system with the production of fatty acid hydroperoxides as the endpoint. The experiments showed that while all the RPE pigments were capable of mediating the photooxidation of fatty acids, lipofuscin had the highest constitutive reaction rate, and thus may be responsible for increasing the basal level of oxidative stress in the RPE and possibly the choroid. This work has been reported separately [19].

We have also continued to characterize the biophysical aspects of the melanosome that contribute

to its interaction with light and with cellular substrates. This work has extended our earlier quantitative analysis of the mechanisms and effects of melanosome photodisruption by pulsed laser radiation [5,6]. In the current phase, we have determined the density of bovine and baboon RPE melanosomes, and incorporated this physical parameter into a simple model that predicts the critical disruption temperature threshold of the melanosome. A report of this work has been published [31]. Related to this work has been the measurement of the optical properties of the RPE melanosome. The refractive index of melanin released from hydrolyzed melanosomes has been experimentally measured, and the scattering and absorption coefficients have been calculated from measurements of transmission and reflection from the melanin samples using the Inverse Adding-Doubling Algorithm. A preliminary report of this work has appeared [32], and a detailed report is in press [33]. Details of these various investigations are given below.

## METHODS

### *Preparation of pigment granules*

Melanosomes (MS) were prepared from human and bovine eyes. Human eyes were kindly donated by the Eye Bank of The Moscow Institute of Eye Microsurgery (Moscow, Russia), and were from donors aged from 40 to 65 years who were free of any ophthalmological disorders. The lipofuscin (LF) granules and melanolipofuscin (MLF) granules were obtained only from human eyes. Human eyes were processed for MS, LF, and MLF by a method modified from that described by Boulton and Marshall [34]. RPE cells obtained from the eyes were sonicated for 60 sec at a frequency of 22 kHz and maximal resonance. The RPE cellular debris was removed by centrifugation at 60 x g for 10 min. The resulting supernatant was then centrifuged at 6000 x g for 10 min. The second centrifugation precipitated the pigment granules, which were resuspended in 0.3 M sucrose, layered on a sucrose density gradient (molarity range: 2.00:1.80:1.60:1.55:1.50:1.40:1.20), and centrifuged at 103,000 x g for 1 hr. The band containing the LF granules was localized between the 1.20 M and 1.40 M sucrose layers, the MLF granules band was between the 1.55 M and 1.60 M layers, and the precipitate contained the melanosomes. The fractions were isolated and centrifuged once more in the sucrose density gradient. The resulting purified granules were washed in 0.1M phosphate buffer several times to remove sucrose. The granules were stored at -20° C until use.

Bovine eyes were obtained freshly in San Antonio, Texas, from local slaughterhouses. Baboon eyes were provided by the Southwest Foundation for Biomedical Research, also in San Antonio. Only melanosomes were prepared from the bovine and baboon tissue. After dissecting away the anterior segments, and removing the retinae, the remaining eyecups were rinsed with tris-buffered saline. The RPE and choroid were removed from the eyecups, placed in 0.25M sucrose in plastic centrifuge tubes, and vigorously vortexed for 30 sec to dislodge RPE cells. The large pieces of choroid were

removed from the centrifuge tubes and the material left behind was sonicated for 30 sec at maximal power. The tubes were placed in swinging-bucket rotors and centrifuged at 2500 x g for at least 10 min. The resulting pellet was resuspended in 10 ml of 0.25 M sucrose, leaving behind any large clumps of tissue, and spun again at 2500 x g for 10 min. This second pellet was resuspended in about 10 ml of 0.1 M sucrose, layered on top of 2 M sucrose, and centrifuged at 2500 X g for 30 min. The resulting separation resulted in a clear, upper fraction and a "fuzzy" interface, which were both discarded. The lower fraction, which contained "light" (round) melanosomes was removed and saved. The pellet contained the "heavy" (ellipsoid) melanosomes. The lower fraction containing the light melanosomes was further purified by diluting it 1:1 with distilled water and centrifuging at 8000 x g for 20 min. The melanosomes were resuspended in 0.25 M sucrose and stored at -20° C. Before use, the melanosomes were spun and resuspended twice in 0.1 M potassium phosphate buffer to remove sucrose. All of the experiments reported here with bovine melanosomes used the "light" fraction, however, in previous work using NADPH oxidation as an assay [7], we found no difference in the photochemical reactivity of the "heavy" and "light" fractions.

#### *Measurement of fatty acid peroxidation*

The peroxidation of fatty acids was determined by the reaction of NADPH-dependent glutathione peroxidase with the hydroperoxide products, and the extent of the reaction was measured by the decrease of NADPH absorption at 340 nm [35]. The production of thiobarbituric acid reactive substances (TBARS) [36,37] was also used to assay the hydroperoxides. Linoleic acid (LA) and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) (both from Sigma Chemical, St. Louis, MO) were used as the fatty acid substrates.

#### *Linoleic acid reactions.*

For LA reactions, 0.72 mg/ml of LA was mixed with 0.1 M potassium phosphate buffer, pH 7.44, and varying densities of pigment granules. Photooxidation was initiated by exposure to a light source which was, in different experiments, either a Xenon arc lamp, or an Argon ion continuous wave laser. After light exposure, an aliquot containing approximately 140-150  $\mu$ g LA was added to 0.1 M potassium phosphate buffer, pH 7.44, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM GSH (reduced form of glutathione), 200  $\mu$ M NADPH, 1.25 units of glutathione reductase, and 0.6 units of glutathione peroxidase. To check the enzymatic activities, 40-50  $\mu$ g tert-butyl hydroperoxide was added at the end of the measurement period.

#### *DHA reactions.*

For DHA reactions, 70-80  $\mu$ g/ml DHA was mixed with 0.1 M potassium phosphate buffer, pH 7.44, 1 mM EDTA, 1mM GSH, 0.7 units GSH reductase, 0.4 units of GSH peroxidase, 200  $\mu$ M NADPH, and varying densities of pigment granules. The sample was divided into two equal parts, one for light exposure, and one for dark control.

#### *TBARS reactions.*

TBARS was determined in DHA samples prepared in 0.1 M potassium phosphate buffer, pH 7.44, 110-130  $\mu$ g/ml DHA, and varying densities of pigment granules. The sample was divided into equal parts for laser exposure and dark control. Following the exposure period, the samples were mixed with 15% trichloroacetic acid and 30 mM thiobarbituric acid and boiled at 100° C for 15 min. After the samples cooled to room temperature, the absorbance at 532 nm was measured.

#### *“White light” exposure.*

Samples were exposed for 10 to 20 min to the broadband output of a 150 W Xenon arc lamp. The



sample irradiance achieved with this system was about 112 mW/cm<sup>2</sup>. Samples were mixed continuously during exposure.

#### *Laser exposure.*

A continuous wave, Argon ion laser (Coherent Medical Systems Model 920) was used to generate blue-green light to excite pigment reactions. The laser was used in a mixed output mode so that both 488.1 nm and 514.5 nm wavelengths (in a ratio of ~45:55 percent) reached the sample. The beam was expanded into an approximately 0.5 cm diameter beam in order to irradiate the entire sample. Sample irradiance was adjusted as required from 50 to 1500 mW/cm<sup>2</sup>, and was monitored continuously during the exposure by means of a prism beamsplitter which deflected a portion of the beam into a power detector. Laser power measurements were made with a Molelectron EPM-1000 meter fitted with a PowerMax 3 head (Molelectron Detectors, Inc, Portland, OR).

#### *Preparation of fluorescent-sensing probe*

The oxidation-sensitive probe, 2'-7'-dichlorofluorescein (DCFH), was obtained from Molecular Probes (Portland, OR). This probe has the property of being nonfluorescent when chemically reduced and highly fluorescent when chemically oxidized. DCFH was supplied from the manufacturer as the diacetate ester (DCFH-DA), and a 1 mM stock solution was prepared by dissolving 10 mg in 20ml of methanol. For studies involving cellular uptake, the ester form was hydrolyzed by the cells themselves (see Results). For studies in cell-free experiments, 0.5 ml of DCFH-DA stock solution was hydrolyzed with 2.0 ml of .01N NaOH for 30 min at room temperature [38]. Hydrolysis was stopped by adding 10 ml of 25mM NaH<sub>2</sub>PO<sub>4</sub> (pH=7.5). Working solutions (usually 10  $\mu$ M) of the probes were made by diluting stock solutions with culture medium. Stock solutions of the fluorescent probes were stored under nitrogen at -20°C, and working solutions were prepared fresh each day.

### *Fluorescence microscopy*

RPE cells were plated on Lab-Tek ChamberSlides (Nalge/Nunc) and allowed to attach to the ChamberSlides. Prior to microscopy, the cells were incubated in 10  $\mu$ M DCFH for 1 hr. After incubation, the cells were briefly washed in probe-free medium and cover-slipped. Photooxidative reactions were activated by exposing the probe-labeled RPE cells to the exciter source in an Olympus BX-60 fluorescence microscope. The fluorescein exciter (max=490 nm) and the Texas red exciter (max=582 nm) were used in different experiments. Fluorescence images were captured on a computer frame grabber at timed intervals during exposure to the excitation source. Image analysis was performed on these images with Image-Pro software (Media Cybernetics) by defining a constant Area of Interest (AOI) in the images, and measuring the mean and standard deviation of the AOI pixel intensity in each image. These values were plotted against the time of image acquisition to construct a fluorescence growth curve.

### *HPLC analysis*

DCFH was analyzed by reversed phase high performance liquid chromatography (HPLC) on a Waters Bondapak-C<sub>18</sub> or a Phenomenex Bondclone-C<sub>18</sub> column. DCFH and its derivatives were eluted with 8mM ammonium phosphate (pH 8.0) modified with 60% methanol. The flow rate was 1 ml/min. Fluorescence was measured by a Waters 474 detector with excitation at 488 nm and emission measured at 530 nm.

### *Extraction of probes for HPLC analysis*

For cellular uptake experiments, aliquots of 10<sup>6</sup> RPE cells were incubated in 10  $\mu$ M probe for varying times. After the desired incubation time, the cells were centrifuged and the resulting pellet washed once in probe-free culture medium. The probe contained within the cells was isolated using solid phase extraction (SPE). This technique of sample preparation utilizes retention of sample and

impurities on a sorbent material similar to that used in a reversed-phase HPLC analytical column, followed by selective elution of the sample molecule(s) with a specific solvent. The impurities are retained on the extraction cartridge. Cells were lysed with 500  $\mu$ l of 0.5% Triton X dissolved in Tris buffered saline and then incubated for 10 min. The samples were centrifuged, and the supernatant containing the probe and soluble components of the cytosol were processed with Waters Oasis HLB SPE cartridges using the following protocol: the cartridge was initially washed and the sorbent wetted by the passage of 1 ml each of methanol and distilled H<sub>2</sub>O (in that order). Without letting the cartridge dry, approximately 0.5 ml of the supernatant was passed through the cartridge. Finally, 1 ml of methanol was passed through the cartridge to elute the probe. Aliquots of this eluant were analyzed by HPLC using the conditions described in section 2.7.

#### *Chemical oxidation of probes for estimation of total uptake*

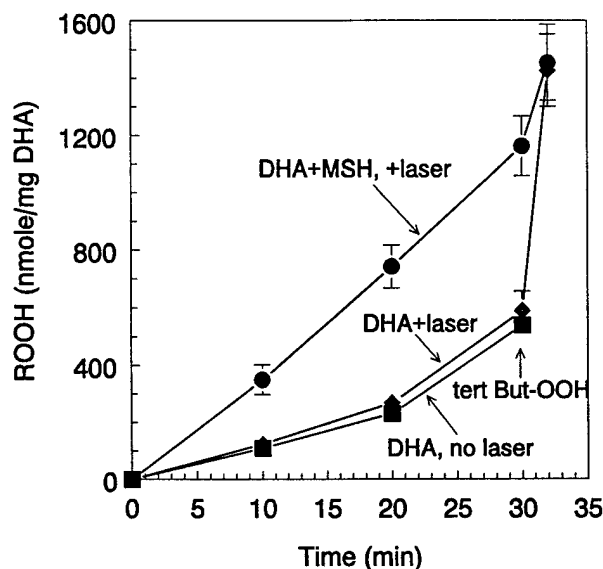
In order to measure total probe content in the uptake experiments, after extraction from the RPE cells, the sample was subjected to chemical oxidation to convert all probe molecules to the oxidized, highly fluorescent moiety. This was accomplished by combining the eluant obtained from SPE extraction with 5- $\mu$ l each of horseradish peroxidase (HRP, 5 mg/ml) and H<sub>2</sub>O<sub>2</sub>, (20 mM) for 30 min at 37° C [38]. These oxidized samples were then analyzed by HPLC with fluorescence detection.

## RESULTS

### I. Photochemical Properties of Isolated RPE Melanosomes

#### *Photooxidation in the absence of pigment granules.*

The fatty acids, LA and DHA, have negligible optical absorbance in the visible spectrum; therefore, when an exposure source with output restricted to the visible wavelengths was used, such as the Argon ion laser, there was very little photooxidation of the fatty acids in the absence of pigment granules. In the experiment presented in Figure 1, the peroxidation rate of DHA alone, exposed to



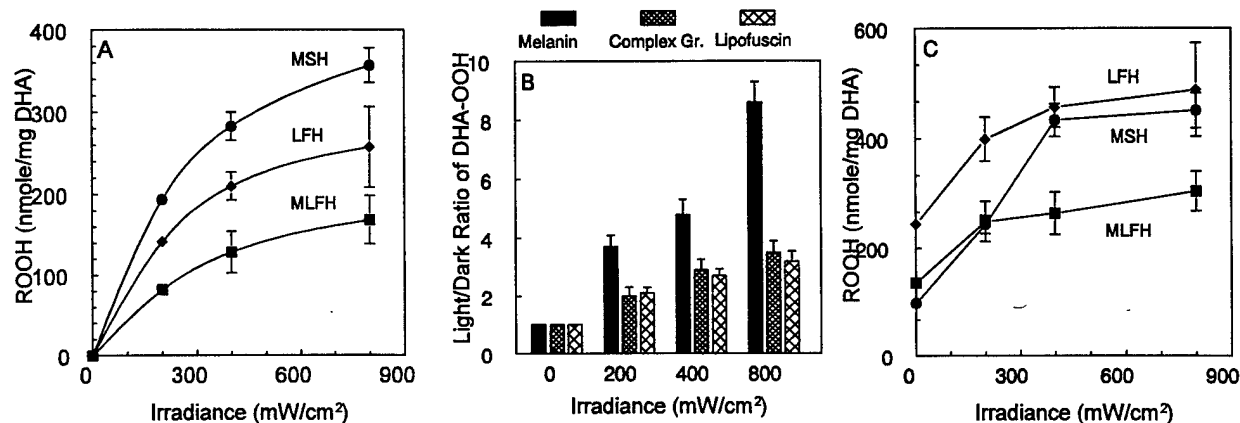
**Figure 1.** The kinetics of DHA peroxidation by Argon laser exposure in the presence and absence of human melanosomes (MSH). When present, human melanosomes were added at a density of  $2.2 \times 10^7$  gran/ml. The reaction was monitored by the oxidation of NADPH coupled to GSH peroxidase. Results were converted into nmole ROOH per mg initial DHA. See text for additional experimental details. Filled squares: autooxidation of DHA in the absence of melanosomes or laser; filled diamonds: DHA solution exposed to laser but without melanosomes; filled circles: DHA solution with melanosomes and exposed to laser output for the durations indicated. At 30 m, t-butylOOH was added to reaction mixture to test activity of GSH peroxidase assay system.

the blue-green output of the Argon laser, was virtually identical to that of unexposed DHA kept in the dark for an equal amount of time. In contrast, when human melanosomes were present at a density of  $2.2 \times 10^7$  gran/ml in the reaction mixture, the amount of photooxidized DHA increased linearly with the duration of the laser exposure (Figure 1). LA was also photooxidized by the laser when melanosomes were present in the reaction mixture (data not shown), but the extent of the reaction was considerably less than with DHA.

*Comparison of the photooxidizing capacity of the RPE pigment granules.*

The three types of pigment granules, MS, LF, and MLF, extracted from human RPE cells were compared with respect to their ability to photooxidize DHA. Reaction mixtures were made up with DHA and either: human MS ( $1.95 \times 10^7$  gran/ml), LF ( $1.72 \times 10^7$  gran/ml), or MLF ( $1.57 \times 10^7$  gran/ml). Aliquots were exposed to the Argon laser for 10 min at  $0.8 \text{ W/cm}^2$ , or kept in the dark as a control. The samples were then analyzed for hydroperoxides by the NADPH-glutathione peroxidase assay. The results are shown in Figure 2A, with the data expressed as the difference between the laser-exposed and dark control samples. The samples with the MS granules contained the largest increase in hydroperoxides after laser exposure, while LF- and MLF-containing samples had lesser increases in hydroperoxides, respectively.

The results of this experiment suggested that MS granules may have the highest photoactivated reactivity towards DHA; indeed, this conclusion is emphasized if the data are replotted as a light/dark ratio (Figure 2B). Two principal conclusions may be drawn from this analysis of the data. The first is that the difference between the light and dark levels of oxidizing activity is the greatest for melanin, while LF and MLF granules show lower ratios of light-to-dark activity. Of the three types of RPE pigment granules, the photoinduced activity of MS granules grew most rapidly with

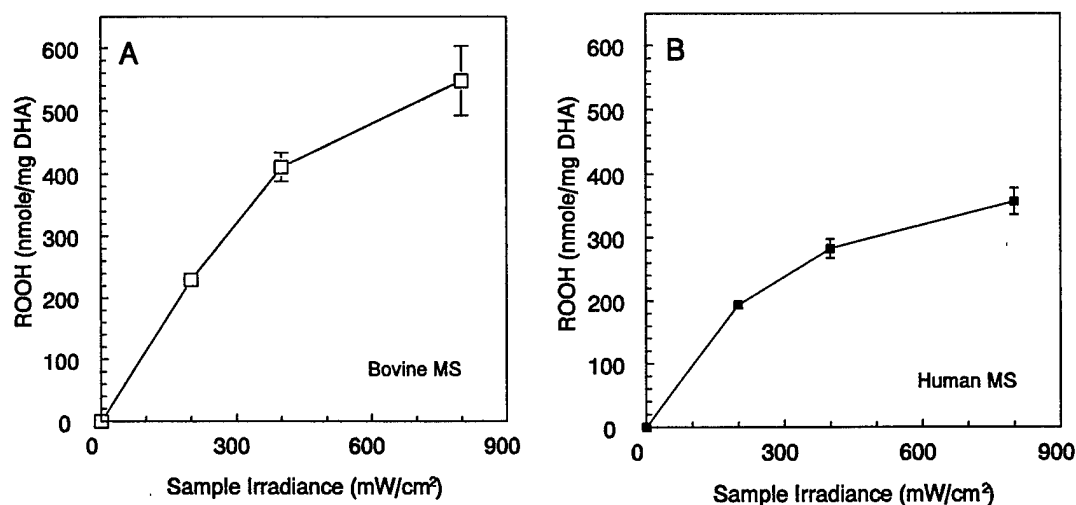


**Figure 2.** Comparison of DHA peroxidation produced by photoactivation of the RPE pigment granules. Argon laser exposure was for a duration of 10 min at the indicated sample irradiances. Each point is the average of two measurements, and the error bars indicate the data range. Abbreviations: MSH, human melanosomes at  $1.95 \times 10^7$  gran/ml; MLFH, human melanolipofuscin granules at  $1.57 \times 10^7$  gran/ml; LFH, human lipofuscin granules at  $1.72 \times 10^7$  gran/ml. A: DHA hydroperoxides produced by photoactivated pigment granules, i.e. the light minus the dark activity. B: The ratio of light to dark activity. C: Total DHA hydroperoxides produced, i.e. the sum of the dark plus light activity. For parts A and C, DHA peroxidation is expressed as nmole of ROOH produced per mg of initial DHA. All reactions were carried out in the presence of 1 mM EDTA.

increasing irradiance. With a sample irradiance of  $800 \text{ mW/cm}^2$ , the light activity of MS rose to nine times its dark level of activity, increasing from a ratio of about 4 with a sample irradiance of  $200 \text{ mW/cm}^2$ . LF and MLF granules, in comparison, only increased from a ratio of 2 up to about 3.5 over this range of irradiance. The second conclusion is that, although the MS granules exhibited the greatest increase in light-induced oxidative reactivity, LF granules possessed the greatest total reactivity towards DHA, i.e. the sum of the DHA hydroperoxides produced during dark and light. This may be appreciated from Figure 2C, which shows total DHA oxidized by the RPE pigment granules during dark and light. The activity of LF and MS granules was approximately equal during laser irradiation, but during the dark LF granules remained reactive while MS granules became quiescent. MLF granules were less reactive overall than were the other two types of pigment granules.

*Dependence of photooxidizing activity of human and bovine melanosomes on irradiance.*

The amount of DHA hydroperoxides formed by photoactivated human and bovine melanosomes depended on their irradiance from a visible light source. Expressed as nmole of hydroperoxide produced per mg DHA, both human and bovine MS showed increasing activity when excited by the Argon blue-green laser emission (Figure 3). Bovine MS were somewhat more reactive than human MS, but because the reaction extent also depended on granule density (see below) this may have simply reflected the uncertainty in the granule counts in the samples. We estimated the error in determining granule counts at  $\pm 20\%$ , and this error may have contributed to some of the difference between the bovine and human granule results.

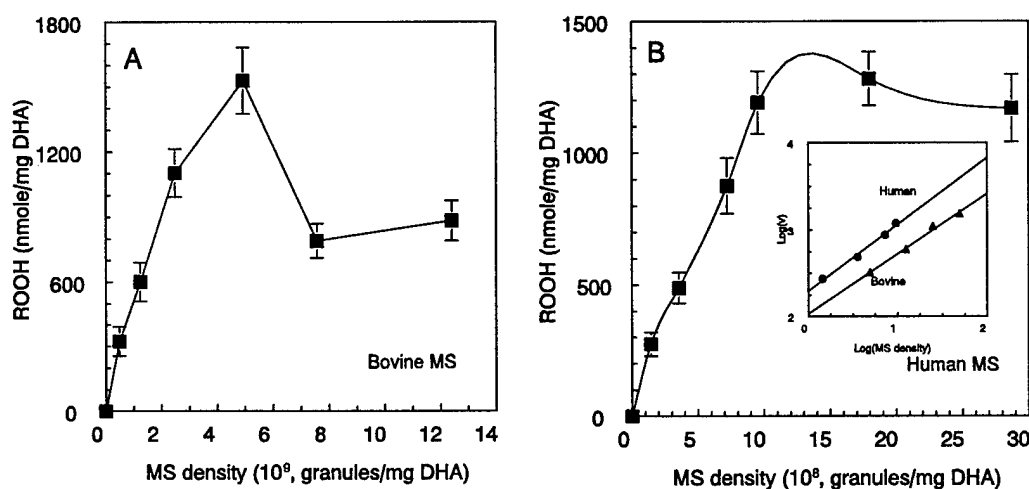


**Figure 3.** Dependence of DHA photooxidation by photoactivated melanosomes on sample irradiance. Samples were exposed for 10 min at various laser intensities. DHA peroxidation was proportional to sample irradiance up to about 1 W/cm<sup>2</sup>. DHA peroxidation expressed as nmole of ROOH produced per mg of initial DHA. A: Bovine melanosomes at a density of  $9.5 \times 10^7$  gran/ml. B: Human melanosomes at a density of  $2.5 \times 10^8$  gran/ml.

*Dependence of photostimulating activity on granule density.*

Photostimulation of DHA hydroperoxide formation was measured for granule densities from  $10^8$  to  $10^9$  gran/ml for bovine MS (Figure 4A), and  $10^7$  to  $10^8$  gran/ml for human MS (Figure 4B). At lower

densities, the photooxidation of DHA was negligible, and at higher densities, saturation and even a reduction in the reaction extent was apparent. As noted above, some of the absolute difference in the results with human and bovine MS may be attributable to inaccuracies in granule counts. Nevertheless, the pattern of increasing photooxidation of DHA with increasing granule density up to a maximum density, followed by a reduction of effect at high granule densities was similar for both human and bovine granules.



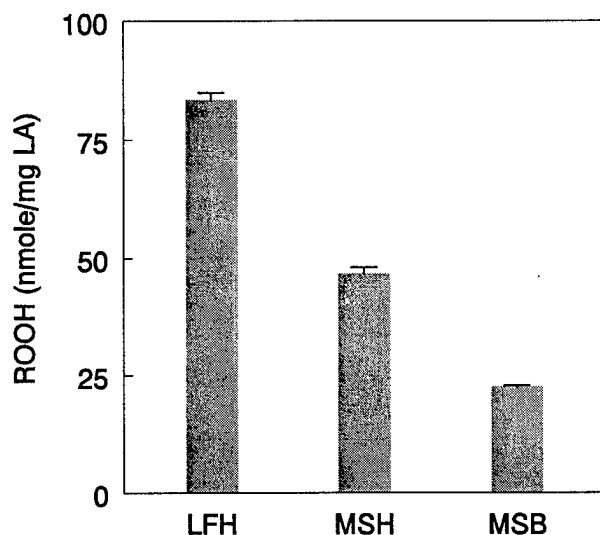
**Figure 4.** Dependence of DHA photooxidation on density of melanosomes. DHA reaction mixtures were prepared with varying densities of bovine (A) or human (B) melanosomes and exposed to the Argon laser at a sample irradiance of  $800 \text{ mW/cm}^2$  for 10 min. ROOH assayed by NADPH coupled to GSH peroxidase as described in text. DHA peroxidation expressed as nmole of ROOH produced per mg of initial DHA. Inset in panel B: plot of relative  $\log(V)$  vs  $\log(\text{granule density})$ . Slope for human MS is 0.77 ( $r=0.995$ ) and for bovine MS is 0.70 ( $r=0.995$ ).

#### *Photooxidation of linoleic acid.*

LA was also photooxidized by light-stimulated pigment granules, although the reaction extent was lower than with DHA. In the data shown in Figure 5, the production of linoleic hydroperoxides is expressed as the difference between the light-stimulated and dark (control) reactions, normalized to a granule density of  $10^7$  gran/ml. Photostimulation was with the Argon laser blue-green emission.



Human lipofuscin was the most reactive toward LA, followed by human MS and bovine MS granules, in that order. A limited supply of LF granules prevented the examination of the dependence of photooxidation of LA on LF granule density and irradiance, as was done with MS granules.

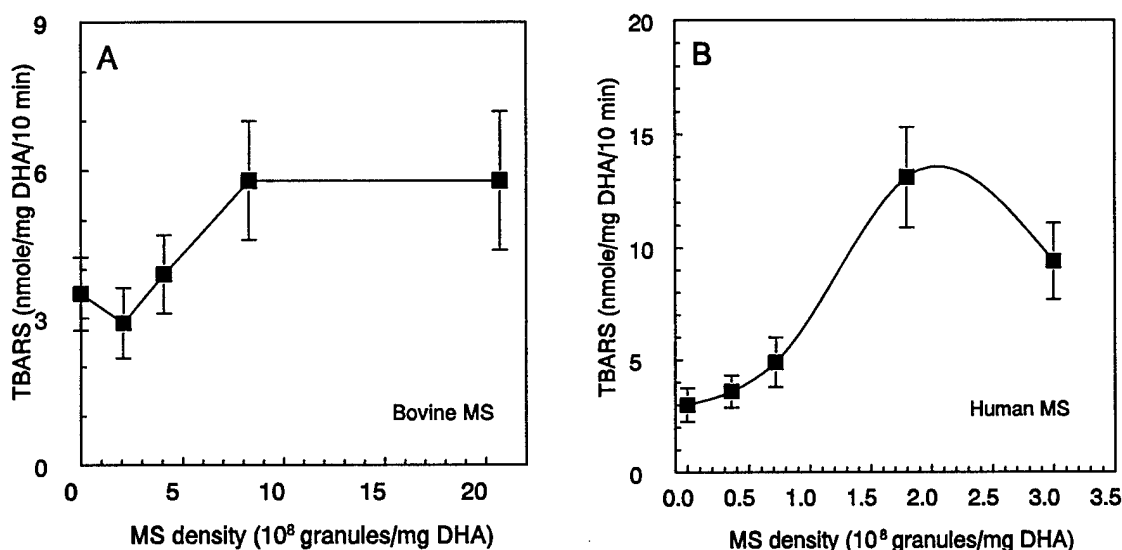


**Figure 5.** Photooxidation of linoleic acid by laser-stimulated RPE pigment granules. Reaction mixtures were made up with linoleic acid and lipofuscin or melanosomes and exposed to the Argon laser for 10 min at a sample irradiance of 1.58 W/cm<sup>2</sup>. Assay for ROOH as described in text. Abbreviations: LFH, human lipofuscin granules; MSH, human melanosomes; MSB, bovine melanosomes.

#### *Effect of broadband light.*

All of the previous experiments were carried out with narrowband or laser sources. The effect of broadband (white) light in photoactivating pigment granules was also determined. In general, broadband light, delivered at a similar irradiance as the laser emission, was much less effective in

exciting the photooxidative reactions. In fact, when a long-pass filter with a cut-on wavelength of 420 nm (Oriel No. 59480) was used in front of the Xenon arc lamp, there was negligible photooxidation; therefore most of the effects described here for the broadband source were likely due to UVA emissions from the lamp (i.e. the wavelengths not passed by the 420 nm longpass filter; sample irradiance due to UVA was estimated at about 20 mW/cm<sup>2</sup> for this lamp). For these measurements, the TBARS assay was used to measure peroxidative breakdown products of DHA. For both bovine (Figure 6A) and human (Figure 6B) MS, there was stimulation of TBARS products when the granules were present at a density up to 10<sup>9</sup> gran/mg DHA for bovine MS and up to about 10<sup>8</sup> gran/mg DHA for human MS.

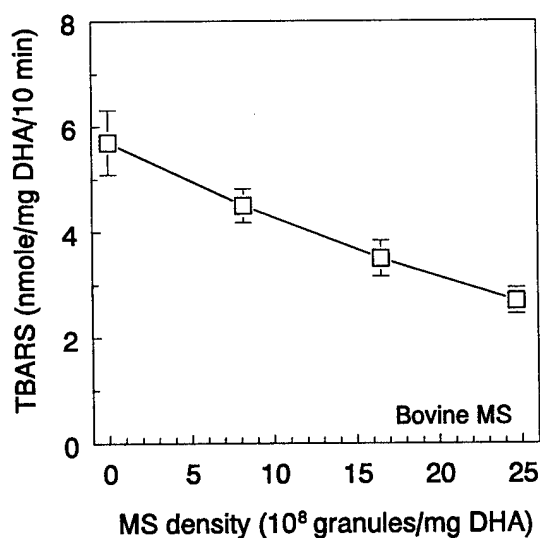


**Figure 6.** Photooxidation of DHA by broadband light. Reaction mixtures were prepared containing DHA and bovine (A) or human (B) melanosomes at the indicated densities, and exposed to the broadband (unfiltered) output of a 150 W Xenon arc lamp. Sample irradiance was 112 mW/cm<sup>2</sup>. Data shown are the average of 10 and 20 min exposures. DHA hydroperoxides were assayed as TBARS. Results are shown as nmole of TBARS produced per mg initial DHA.

#### *Activity of Melanosomes in the presence of a photosensitizer.*

In order to determine the relative reactivity of MS granules compared to exogenous photosensitizers

known to produce reactive oxygen species, the TBARS from DHA photooxidation were determined in the presence of a mixture of methionine and riboflavin illuminated by blue light. For these experiments, the light source was the Xenon arc lamp with the output filtered with a 400 nm bandpass filter (Oriel No. 59820). With the methionine-riboflavin photosensitizer present, this weak blue light source was able to produce an increase in TBARS compared to the nonirradiated control, indicating the production of reactive oxygen species, probably superoxide anion [39]. When bovine MS were present, however, the amount of TBARS produced decreased in an amount proportional to the density of the MS granules in the reaction mixture (Figure 7). The effect of human MS in this reaction was not determined because of insufficient experimental material.



**Figure 7.** Effect of increasing bovine melanosome density on DHA peroxidation by a photosensitized system of riboflavin/methionine. Photoactivation was with 400 nm light at a sample irradiance of 5.3 mW/cm<sup>2</sup>. See text for additional experimental details. Peroxidation was assayed as TBARS. Addition of bovine MS inhibited production of TBARS.

## II. Physical and Optical Properties of Isolated RPE Melanosomes and Melanin

### *Density measurement of melanosomes*

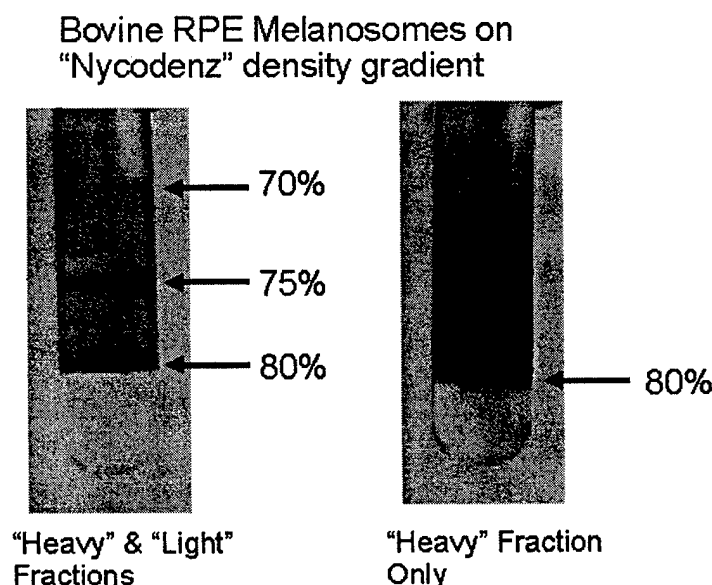
Density, along with the absorption coefficient, water content, and specific heat, figure into the relationship between laser exposure and melanosome disruption, which is one of the major components of laser retinal injury. Calculations using these parameters have been used to estimate the damage thresholds for skin melanin [40] and RPE melanin [5,6]. For example, for a given target tissue, the radiant exposure,  $H_{th}$  ( $J/cm^2$ ), required to reach the damage threshold is given by

$$H_{th} = (\rho C \Delta T) / \mu_a \quad (1)$$

where  $\rho$  is the tissue density ( $gm/cm^3$ ),  $C$  is the tissue specific heat capacity ( $J/cm^3 \cdot ^\circ C$ ),  $\Delta T$  is the temperature increase from ambient required to reach the damage threshold, and  $\mu_a$  is the absorption coefficient. The values used for melanosome density, however, were available previously, but were extrapolated from estimates of water content derived indirectly. Recently the density of bovine and baboon RPE melanosomes has been measured directly by density gradient centrifugation. During centrifugation, particles experience acceleration until they encounter a medium of similar density. At that point, there is no net force exerted on them, and they remain in a stationary band at that position in the medium. By indirectly determining the density of the surrounding medium from its refractive index, the density of the particles is deduced.

Melanosomes were isolated from sonicated bovine RPE cells and purified by centrifugation through sucrose solutions [3]. These partially purified melanosomes were then layered on a density gradient medium contained in a swinging-bucket rotor and spun at 10,000 rpm (average RCF = 12,622 x g) for 1 hr. To avoid osmotic changes in the water content of the melanosome, which would affect the apparent density of the particles, we used the nonionic density medium, Nycodenz [41], for these

experiments. The density gradient was formed in steps by layering decreasing concentrations of Nycodenz in the centrifuge tube starting with 80% and decreasing to 60%. With partially purified melanosomes, two bands were formed after centrifugation, one at the interface between 70% and 75%, and one between 75% and 80%, corresponding to densities of 1.38 and 1.41 gm/cm<sup>3</sup>, respectively (Figure 8, left-hand tube). Thus, in the bovine RPE, there are apparently two populations of melanosomes: one heavy and one light, possibly corresponding to the ellipsoid and spherical morphological subtypes. We also analyzed a more purified preparation of bovine melanosomes. When this preparation was analyzed on the Nycodenz gradient, most of the melanosomes banded at the 75/80 percent interface, corresponding to the heavier population of melanosomes (Figure 8, right-hand tube). Melanosomes isolated from baboon RPE cells were also subjected to a similar analysis, and were found to have a similar density. In upcoming work, we plan to utilize this density data to extend our earlier estimates of RPE disruption threshold and internal absorption coefficient.



**Figure 8.** Photographs of melanosomes separated by density centrifugation on Nycodenz gradients.

### *Optical properties of melanosomes: absorption and scattering coefficients*

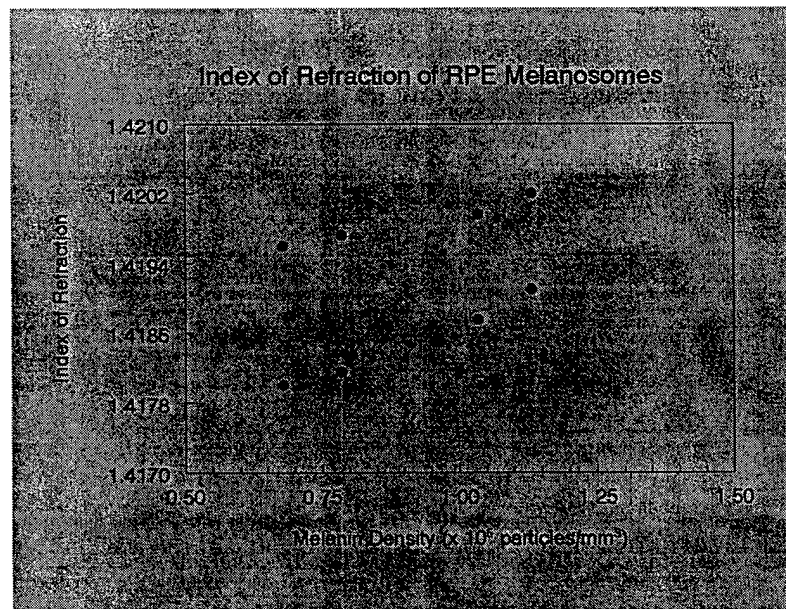
There are few published values for the absorption coefficient of skin or RPE melanosomes, and the values reported range from a high of about  $9600 \text{ cm}^{-1}$  to a low of about  $80 \text{ cm}^{-1}$  at mid-visible wavelengths [6,40,42]. Some of this variation may be due to species variation, sample preparation, measurement technique, and the oxidation state of the sample. Because of the difficulty of solubilizing melanin, e.g. boiling for protracted times in strong acid or base, many measurements have been made of suspensions of the material. In this state, it is difficult simply by measuring the bulk optical transport through the suspension to separate the effects of light scattering from light absorption. Therefore we made an attempt to measure absorption and scattering coefficients separately. Bovine melanosomes were isolated as described in Methods, stripped of the melanoproteins by moderate acid hydrolysis, i.e. incubation in 1 M HCl at 60 °F for eight hours, and dispersed as a fine suspension in distilled water. The samples were placed in a glass cell held between two integrating spheres. Total diffuse reflectance and transmittance measurements were taken at several visible wavelengths using Argon ion (488 and 514.5 nm), doubled Nd:YAG (532 nm), and HeNe (633 nm) laser sources. Anisotropy of the melanin samples was also measured, using a goniometer and a HeNe laser as the light source, and was found to be 0.94 at 633 nm. These data were used as input parameters for the Inverse Adding Doubling algorithm [43] (IAD code provided courtesy of Dr. Scott Prahl of the Oregon Medical Laser Center, available on the Internet at: <http://omlc.ogi.edu/software/>). Some representative values for the absorption coefficient,  $\mu_a$ , and scattering coefficient,  $\mu_s$ , are shown below:

Wavelength (nm)	$\mu_a \text{ (cm}^{-1}\text{)}$	$\mu_s \text{ (cm}^{-1}\text{)}$
488	11.7	642
532	8.7	567

Although the values reported here for the absorption coefficient are lower than those reported elsewhere, it should be noted that if an estimate of total optical penetration depth is made using the sum of  $\mu_a$  and  $\mu_s$ , then values of 600 to 700  $\text{cm}^{-1}$  are obtained, which are comparable to previous estimates of the bulk optical transport of melanosomes.

### *Index of refraction*

We have also measured the index of refraction of a melanosome suspension. In the mid-visible wavelength range, the index of refraction ranges between 1.41 and 1.42, increasing slightly with increasing number of melanosomes (Figure 9) (more details on the optical properties of melanin may be found in Sardar et al. [33]). In future work, it is planned to incorporate these data in a model of light interactions with ocular melanin.



**Figure 9.** Index of refraction measured for bovine melanosomes at different particle densities and wavelengths. Triangles: data measured with incident laser wavelength of 633 nm. Circles: data measured with incident laser wavelength of 532 nm.

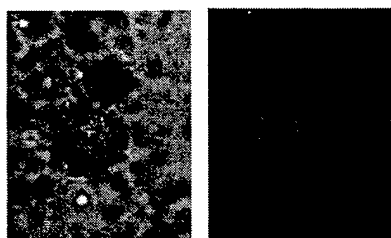
### III. Studies on Intact RPE Cells

#### *Fluorescence microscopic studies of intracellular photo-oxidative stress*

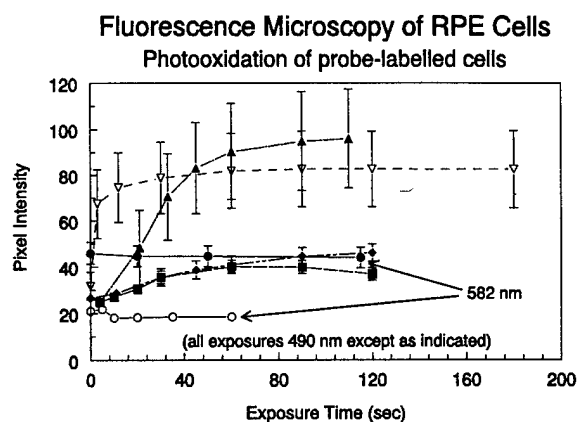
The use of a vital, fluorescent probe (intracellular stain) offered the possibility of obtaining direct visualization of photooxidative stress within the RPE cells, especially the spatial localization of oxidative reactions. Although the early data were encouraging, two factors prevented this imaging approach from yielding the quantitative data desired. One was the difficulty in calibrating the fluorescence signal so that meaningful comparisons could be made between experiments. The second problem resulted from the cells' pigmentation. Many of the primary RPE cell cultures used in these studies were heavily pigmented, retaining most if not all of their melanosome content. We found that the presence of the heavy pigment tended to quench or block the fluorescence signal from the cells. In many experiments, only a bright, fluorescent halo could be observed around the cell. However, one salient observation was made when intracellular fluorescence was clearly observed: the fluorescence was limited to the cytoplasm, and did not extend into the nucleus (Figure 10). This was of interest because it implied that photooxidative reactions occurred in the vicinity of the melanosomes. In these experiments, the photooxidative stress was produced with the exciter of the fluorescence microscope, which was simply allowed to illuminate the cells for various lengths of time. As described in the Methods, two exciter wavelengths were used, one with a spectrum centered at 490 nm and the other at 582 nm. Several cells were imaged clearly enough to measure the relative increase in fluorescence with increasing exposure time. The changes in pixel intensity of a defined AOI in images of 5 RPE cells exposed to the microscope exciter are plotted in Figure 11. These observations indicated that the 490 nm light caused an increase in the intracellular fluorescence, while there was little or no increase when cells were exposed to the 582 nm light. Nevertheless, the limited, qualitative nature of the imaging data forced us to develop a biochemical methodology for the direct analysis by HPLC of the amount of the DCFH probe oxidized by various



wavelengths of light. The results of these studies are described in the following sections.



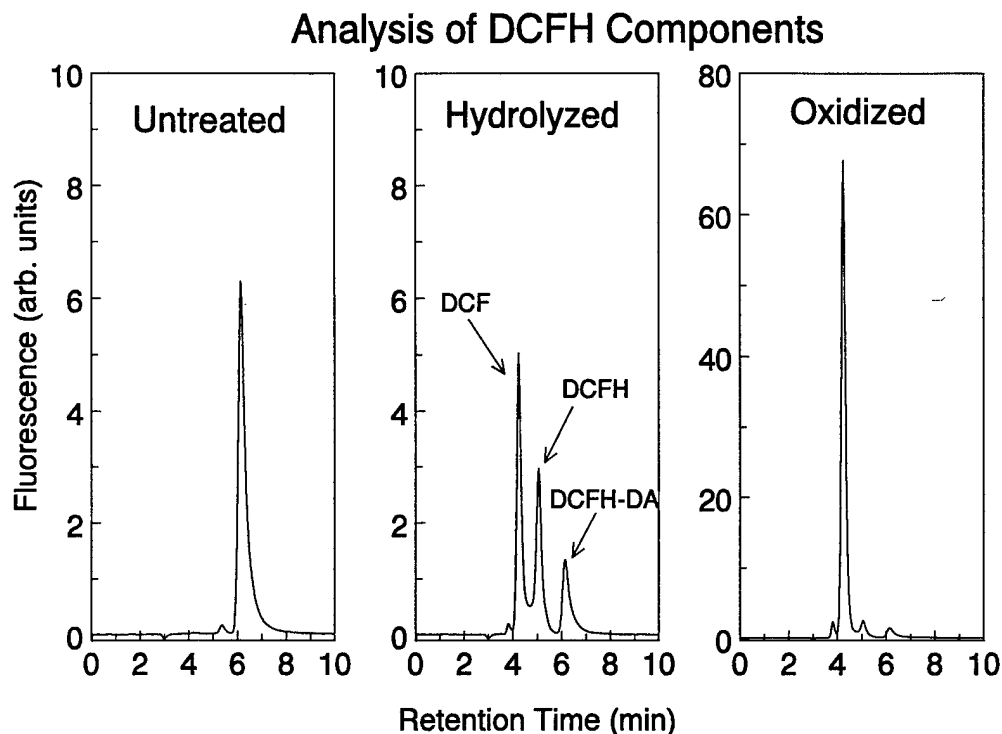
**Figure 10.** Micrographs of DCFH-labeled cells. Left: bright field image. Right: fluorescence micrograph of same cell using fluorescein exciter, showing fluorescence restricted to cell's cytoplasm.



**Figure 11.** Increase in fluorescence of probe-labeled RPE cells continuously exposed to microscope exciter lamp (wavelengths indicated in figure).

#### *DCFH-DA uptake and hydrolysis in RPE cells*

DCFH is supplied as a diacetate ester (DCFH-DA), a chemical form which facilitates its penetration into cells. Once in the cell, nonspecific esterases are thought to cleave the ester from the rest of the molecule [38]. HPLC analysis was used to confirm the uptake and processing of the probe by the RPE cells. DCFH-DA and its two principal metabolites, the hydrolyzed-reduced form DCFH, and the hydrolyzed-oxidized form DCF, exhibited distinct retention times in HPLC analysis (Figure 12). In this figure, the left-hand panel shows a chromatogram of the stock probe, DCFH-DA. This form elutes as a single peak at about 6.5 min under our HPLC conditions. After the probe is chemically hydrolyzed and oxidized, HPLC analysis reveals a single, highly fluorescent peak eluting at 4.25 min (note difference in vertical scale between the right hand panel and the other two). HPLC analysis of DCFH that was chemically hydrolyzed, but not oxidized, is shown in the central panel. The

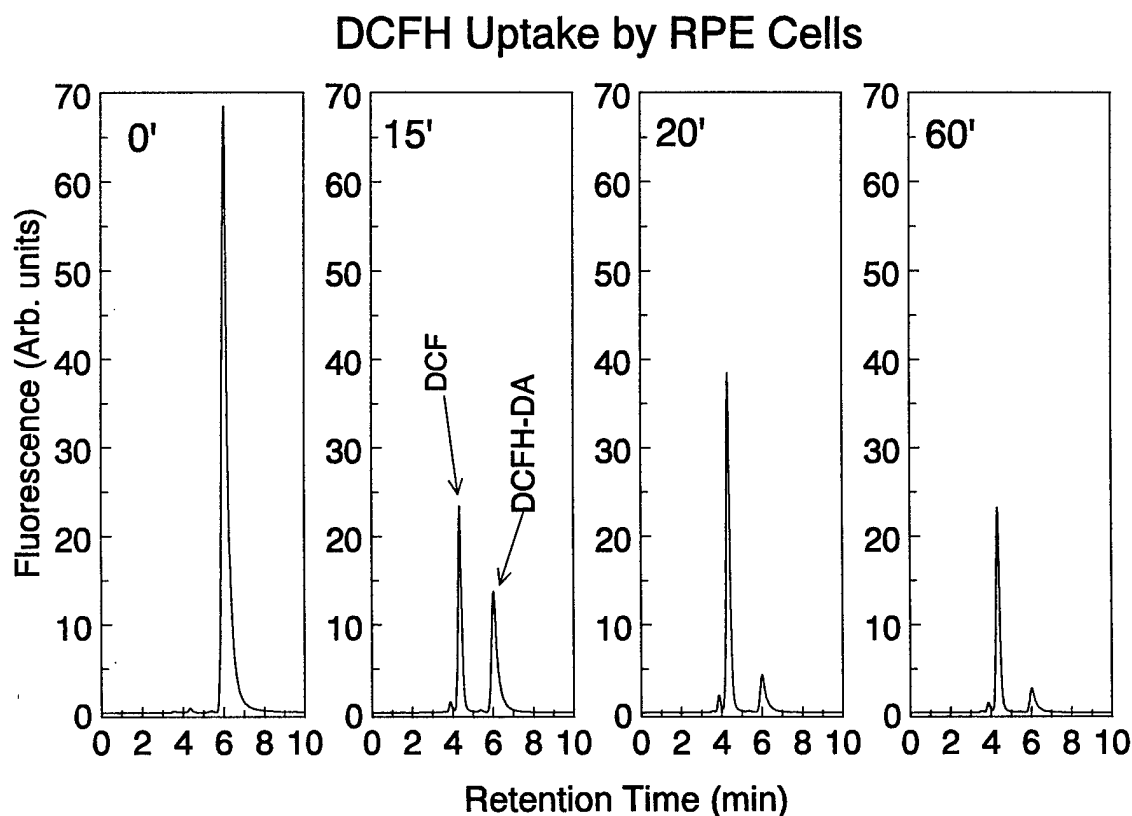


**Figure 12.** HPLC analysis of DCFH components. Left panel: DCFH-DA; Center panel: showing the separation of DCFH-DA, DCFH, and DCF simultaneously injected into the analytical column; Right panel: DCF alone (hydrolyzed and oxidized).

hydrolyzed-reduced form, DCFH, elutes at about 5 min, between DCF and DCFH-DA. Note that all forms of DCFH have some degree of fluorescence, which facilitated the detection of each moiety by HPLC, but the quantal efficiency of the oxidized form is much greater than that of the reduced form.

HPLC analysis demonstrated that, following uptake, the probe is hydrolyzed to DCFH inside the RPE cells. Cells were incubated with a 10  $\mu$ M solution of DCFH-DA, and aliquots were removed for assay at 20 minute intervals, beginning at 0 min of incubation. The sample was extracted using SPE, chemically oxidized to enhance detection of the compounds, and analyzed by HPLC with fluorescence detection. The peaks in the resulting chromatograms were identified by comparison to the known retention times of the identified chemical species shown in Figure 12. The

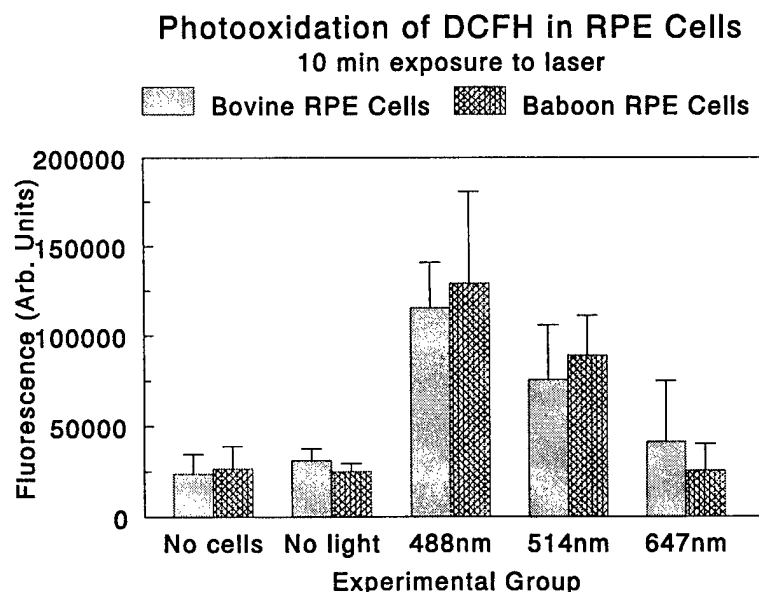
chromatograms obtained at  $t = 0'$ ,  $15'$ ,  $20'$ , and  $60'$  are shown in Figure 13. At time =  $0'$ , all of the probe is present as DCFH-DA. By  $15'$ , probe has accumulated in the cells and is starting to be hydrolyzed. (Note that because these samples have been manually oxidized prior to HPLC analysis, little or no DCFH is found, because all DCFH produced by the action of cellular esterases is oxidized to DCF by our procedure). By  $20'$ , most accumulated probe has been hydrolyzed, and by  $60'$  virtually all detectable probe in the cells has been hydrolyzed. Apparently, the total probe content in the cells declines by  $60'$  either because of efflux from the cell, or metabolic breakdown to a form that is either non-fluorescent or not captured by our isolation procedure.



**Figure 13.** Uptake and processing of DCFH-DA by RPE cells. HPLC analysis, as described in the text, of RPE cell lysates after indicated times of incubation in  $10 \mu\text{M}$  DCFH. Initially (at time =  $0$ ) all probe is present as the diacetate ester. With longer incubation times, the probe is increasingly converted to DCFH, the hydrolyzed form.

### *Photo-oxidation of intracellular probe*

Aliquots of bovine or baboon RPE cells were incubated with 10  $\mu$ M DCFH or DHR123 for 1 hr, and then were either exposed to laser or maintained in the dark as a control. Ten-minute light exposures were made with the 488 or 514.5 nm emissions of the Argon ion laser, or the 647.1 nm emission of the Krypton ion laser. All exposures were quantum-equivalent, delivering  $\sim 4.99 \times 10^{18}$  photons/cm<sup>2</sup>-sec in 10 min. Following the laser exposure, the cells were lysed and the probe isolated from the lysates by SPE. Twenty- $\mu$ l aliquots were injected into the HPLC column and the probe content quantified by fluorescence detection. Laser exposure produced a wavelength-dependent pattern of DCFH photooxidation (Figure 14). In the dark, or in cell-free medium, there was a low level of DCF fluorescence, indicating that most of the probe remained in the reduced form. All three laser wavelengths resulted in DCFH oxidation, but the degree to which the probe was oxidized was clearly wavelength dependent. On a quantal basis, the Argon 488 nm output was the most efficient in producing photooxidation. The results were similar for bovine and baboon cells.



**Figure 14.** Laser photooxidation of DCFH incorporated into cultured bovine and baboon RPE cells. All exposures were quantum-equivalent (see text for details), and produced a wavelength-dependent pattern of oxidation.

## DISCUSSION

### *Photooxidizing activity of rpe pigment granules.*

The RPE of normal human and animal eyes contains pigmented inclusions related to three types of pigment granules: melanosomes, melanolipofuscin and lipofuscin. RPE melanosomes contain the black or brown screening pigment melanin. The "age pigment", lipofuscin, is a heterogeneous aggregation of damaged molecules, rather than a genetically programmed, native product. Complex, or melanolipofuscin, granules are presumably the concretion of melanin and lipofuscin pigments due to the effects of degenerative or aging processes. Although it is generally thought that RPE melanosomes protect ocular tissue against light damage by passively absorbing excess light [44,45] or inhibiting free radical reactions [46], it is known that melanosomes are able to generate toxic superoxide radicals and hydrogen peroxide under illumination [23,47,48] and to promote photooxidation processes in vitro [3-5]. According to some authors these processes may intensify the damaging effect of illumination on RPE and neural retina [49,50], especially with blue light [51,52]. Nevertheless, it is not clear under what particular conditions the melanosomes are able to stimulate versus inhibit free-radical reactions.

Lipofuscin, a heterogeneous aggregation of damaged molecules, accumulates in the RPE so that in elderly persons, the amount of lipofuscin may exceed that of melanin by five to ten times [14]. There is also an age-related increase in the quantity of the hybrid compound, melanolipofuscin, contained in granules within RPE cells. Lipofuscin consists of molecules so seriously altered that they can not be degraded by native enzymes [53,54]. RPE lipofuscin apparently represents the lifelong accumulation of lysosomal residual bodies containing the end products of the phagocytosis of photoreceptor outer segments [55-57]. High levels of lipofuscin are thought to exacerbate the aging process and have been associated with a number of pathologies which include both ocular and

systemic disorders. Indeed, in some cases of retinal degenerations, it has been shown that the greatest lipofuscin accumulation in the RPE was associated with the greatest photoreceptor loss in the overlying retina [58]. Lipofuscin granules are able to photosensitize the generation of superoxide radicals [28] and singlet oxygen [16]. Although UV irradiation effectively excites free radical activity of LF *in vitro* [16,17], there is little information about *in vivo* conditions that cause lipofuscin granules to stimulate free-radical oxidative reactions, namely intraocular wavelength and intensity of light, the critical concentration of lipofuscin, the effect of the concentration of oxidizable substrates, oxygen saturation, etc. The exact mechanisms, too, by which lipofuscin might produce damage *in situ* are not well understood [17,18,29].

The work reported here has confirmed our previous findings that all of the RPE pigments have photooxidizing activity [1-5,7,48], as well as earlier reports of the photochemical activity of melanins [24,25,59-61]. The present work has extended the previous findings by focusing on the photochemical activity of the RPE pigments towards fatty acids, especially docosahexaenoic acid, which in the retina is an essential fatty acid comprising 25% or more of the polyunsaturated fatty acids in the rod photoreceptors [30,62] and has been implicated in normal visual development and function [63,64].

A major finding reported here is that while all of the RPE pigment granules have photoinducible oxidizing activity towards the polyunsaturated fatty acids DHA and LA, there are differences in the dark activity levels of the pigments. Specifically, while RPE melanosomes are the most active in the photooxidation of DHA, in the dark this reaction is essentially quiescent. LF granules in contrast are activated much less strongly than are MS granules, but have a much higher dark reactivity. MLF (complex) granules have activity characteristics similar to LF granules, i.e. they exhibit some photoactivated activity, but also a relatively high dark reactivity. As shown in Figure 2C, the total

reactivities of these pigments relative to each other, i.e. the dark plus light-activated peroxidation of DHA, indicate that LF and light-activated MS granules are approximately equally reactive, while MLF granules are less reactive by half. The effect of light in stimulating the reactivity of MS granules is also clearly shown in Figure 2C, with the greatest increase in reactivity occurring over the physiologically relevant irradiance range from zero (dark) to 400 mW/cm<sup>2</sup>.

#### *Effect of RPE pigment granule density, and light exposure parameters*

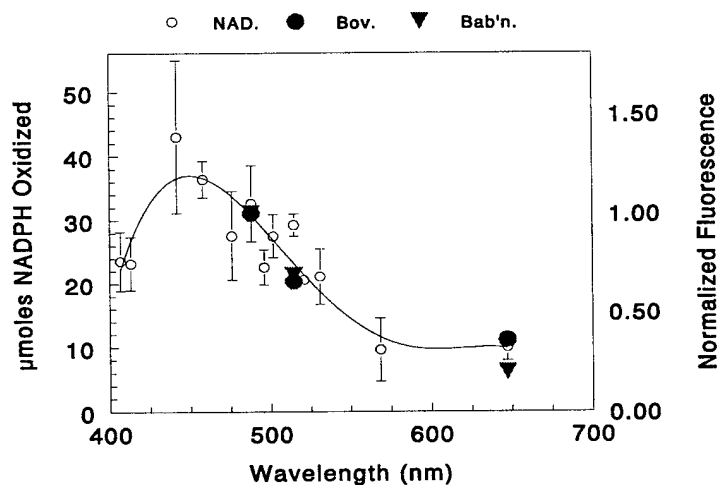
The production of DHA hydroperoxides was stimulated by increasing the sample irradiance by the laser as well as increasing the density of pigment granules, at least up to a saturation point with MS. The saturation observed with increased MS granule density may have been due to self-screening by the high optical density of the MS suspensions, and was similar to the saturation observed in a previous report on the photooxidation of linoleic acid by laser-excited MS granules [4]. The lowest radiant exposure (product of irradiance and time) used in these experiments to produce detectable peroxidation of DHA was about 120 J/cm<sup>2</sup> (0.2 W/cm<sup>2</sup> for 10 min). For the 488 nm and 514.5 nm emissions of the Argon laser, this was visually a very bright light, but was only about 1/8th of the threshold for thermal damage calculated as the equivalent retinal radiant exposure produced by the incident corneal threshold limit allowed by the ANSI laser safety standard [65]). Therefore it was unlikely fatty acid reactions observed in these experiments were due to thermal processes. Also in previous work, we found that the pro-oxidant activity of light-activated melanin was essentially quenched by the presence of antioxidants such as ascorbic acid (vitamin C) and  $\alpha$ -tocopherol (vitamin E) [2].

#### *Significance of the wavelength dependence of the DCFH photooxidation*

Although the intracellular photooxidative conversion of DCFH to DCF has been characterized for only three major laser wavelengths, the results obtained are very consistent with the action spectrum

reported for the photochemical oxidation of NADPH by laser-excited RPE melanin [7]. The oxidation of NADPH in that study was used as an assay for the excitation of reactive free radical sites in the melanin heteropolymer, and the resulting action spectrum exhibited a peak in the visible spectrum between 450 and 500 nm. For comparison, the data shown in Figure 14 has been normalized and superimposed on the melanin action spectrum taken from Figure 1 of reference [7], and replotted in Figure 15. It is clear that photooxidation of DCFH in laser-exposed RPE cells follows a very similar wavelength dependence as that of NADPH with melanosomes.

The localization of DCF fluorescence only in the cytoplasm of the cell argues strongly that the chromophore responsible for this photochemical reaction is one of the RPE pigment granules.



**Figure 15.** Action spectrum of NADPH oxidation by laser-excited melanin (all exposures  $\approx 3.18 \times 10^{21}$  photons/cm<sup>2</sup>/300 sec). Open circles: photooxidation of NADPH, data from reference 7. Superimposed data: normalized fluorescence of DCFH in bovine (filled circles) and baboon (filled triangles) RPE cells, data taken from Fig 14.

Melanin is the most likely candidate; one, because of the overlap of the DCFH photooxidation data with the melanin action spectrum, and two, because lipofuscin and melanolipofuscin, the other two RPE pigment granules, are not found in appreciable quantities in the young bovine eyes used in some



of the present experiments. The baboon eyes were from older animals that may have contained larger amounts of lipofuscin, therefore the contribution of lipofuscin cannot be totally excluded. There was insufficient tissue available from the baboon eyes to analyze for lipofuscin. Nevertheless, because as we have shown lipofuscin is capable of inducing oxidative reactions even in the dark [19], the low level of dark oxidation in our preparations suggests that the contribution of lipofuscin granules was not significant.

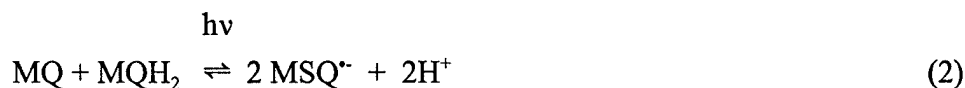
#### *Significance of pigment-mediated photooxidation in the RPE cell*

The oxidation of DCFH in laser-exposed RPE cells serves as a beacon of oxidative stress. It is known that this probe can be oxidized by several reactive oxygen species [38]. These reactive oxygen species are produced by photochemical reactions, and indeed have been reported in studies on light-activated melanin [22]. In view of the photoreactivity of the isolated granules, as well as the reports in the literature concerning oxidative damage to physiological molecules due to light-activated RPE pigment granules [1,3,16,18,19,25], one may speculate that the origin of this oxidative stress is the free radical activity induced in the pigment granules during light exposure. Unless the RPE cells have antioxidants that can quench reactive species produced directly or indirectly by light-activated pigment granules, cellular damage will ensue. The cumulative effects of such damage may be the basis of some of the age-related retinal degenerations. The melanosome also breaks down in age, leading to the accumulation of melanolipofuscin granules along with the lipofuscin granules [14]. The breakdown of the melanosomal structure has been shown to increase its photochemical reactivity [5]. Taken together, all of these observations lead to the general conclusion that the RPE pigments may play dual roles in the eye: protecting against light damage as long as sufficient antioxidants such as ascorbic acid are present to quench the light-excited pigment radicals, but promoting light damage in the event of antioxidant depletion or inability to recycle antioxidants. Examination of these related aspects of RPE cell metabolism may yield new insights into the

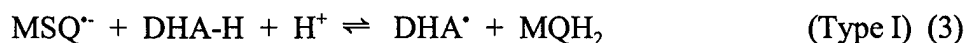
prevention of oxidative stress in the retina and its supportive tissues.

### *Possible reaction mechanisms*

The rate dependence of the production of DHA hydroperoxides on the density of MS granules prior to the saturation of the reaction (c.f. inset in Figure 4B) was found to have a slope close to one, suggesting that the reaction was first order with respect to MS granules. Based on this and on the assumption that molecular oxygen is present in excess, it is likely that the reaction of photoexcited melanin proceeds according the mechanism(s) proposed by Rozanowska et al. [25]. The probable primary reactive species are the quinone (MQ), semiquinone (MSQ<sup>•</sup>), and hydroquinone (MQH<sub>2</sub>) groups of the hydroxyindole subunits of the melanin heteropolymer. The effect of visible light is to push the equilibrium between the quinone, hydroquinone, and semiquinone species to the right so that the occurrence of the semiquinone form is favored:



This is presumably the only light-induced reaction. The semiquinones may react with directly with DHA in a Type I (free radical) reaction or through a Type II reaction involving an oxygen radical intermediate [66,67] as follows:



where DHA-H represents the polyunsaturated fatty acid and DHA<sup>•</sup> the alkyl radical, or



where the reaction of the semiquinone radical with oxygen produces superoxide anion. Following this(ese) reaction(s), hydroperoxides of the fatty acids may be produced in the following reactions:



where DHA<sup>•</sup> is formed by abstraction of a hydrogen atom by the perhydroxyl radical, HOO<sup>•</sup>.

Although this conjugate acid may only represent 1% of the total superoxide at physiological pH, it is relatively efficient at producing fatty acid hydroperoxides, especially if hydroperoxides are already present [68]. Interaction of the alkyl radical with oxygen forms a peroxy radical:



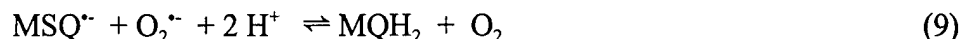
A chain reaction between the peroxy radical and the PUFA ensues, producing the hydroperoxide products:



The dienes produced by these processes can be conjugated or non-conjugated, depending on whether a Type I or Type II reaction occurred [69], but our methodology did not include such an analysis. Based on energetic and rate considerations, it is likely that a majority of the hydroperoxides were produced by a Type II reaction.

#### *Interaction of melanin with a photosensitizer.*

When superoxide was generated by the photosensitized system of riboflavin-methionine, we observed that the peroxidation of DHA was diminished by the presence of melanin. This result could have been due to the screening of the riboflavin from photic stimulation. Another possibility is that the superoxide generated by the photosensitizer reacted with the melanosomes instead of the DHA. That the reaction of superoxide with melanin is favored is indicated by the inhibition of nitroblue tetrazolium reduction by superoxide in the presence of melanin [70], and also by its high rate constant ( $>10^4 \text{ M}^{-1}\text{-s}^{-1}$ ) [71,72]. Melanins generally exist in an oxidized form [73], probably because of interaction with oxygen. Generally, the oxidized forms of melanin (the quinone and semiquinone forms) react with superoxide as shown:



If a large excess of  $\text{O}_2^{\cdot -}$  is produced, for example, in the photosensitized riboflavin-methionine experiment, then reactions (8) and (9) will be pushed to the right, consuming the superoxide in the process and reducing melanin. The reaction of melanin hydroquinones with continuously-produced superoxide may lead to regeneration of the melanin semiquinone radical, as proposed by Jarabak et al. [74] for the redox cycling of o-quinones in the presence of superoxide. In addition, if the oxidation potential of eumelanin is similar to that of synthetic dopa-melanin, recently determined by cyclic voltammetry to be less than +100 mV [75], then the redox cycling of melanin would be thermodynamically favored over oxidation of PUFA, which typically occurs at an oxidation potential of +600 mV [76]. Nevertheless, the precise mechanism of the interaction of melanin and exogenous photosensitizers remains to be determined.

#### *Reactivity of LF granules in the dark*

Several recent studies have indicated that human LF granules contain free radical and other transient species inducible by UVA exposure which produce reactive oxygen species and induce lipid peroxidation [16-18]. We now add the finding that even in the dark, human LF granules are reactive towards polyunsaturated fatty acids. Although one component of human LF has been chemically characterized at present [77], LF granules isolated from RPE cells are not homogeneous and contain several other, uncharacterized components [16]. Their dark reactivity evidently arises from other than a photochemical process, possibly involving reactions catalyzed by labile hydroperoxides accumulated during the degradation process which gives rise to the LF granules. These labile hydroperoxides readily undergo thermally-dependent, homolytic decomposition to free radical products, which catalyze in the dark the DHA peroxidation. In any event, the constant

reactivity of LF granules, if it occurs *in situ*, would be a major factor contributing to oxidative stress in the RPE.

*Implications for Origins of Oxidative Stress in the Retina-RPE Complex.*

Currently, we can only speculate on the significance of the reactions observed with isolated RPE pigment granules for the generation of photooxidative stress in the RPE and choroid. If oxidative reactions with polyunsaturated fatty acids were initiated by photoactivated RPE melanin and lipofuscin granules, then damage could occur to the RPE cell membranes, and possibly to the photoreceptors with their large content of membranous disks in the outer segments. The relatively high oxygen tension in the choroidal tissue would appear to amplify the risk of lipid peroxidation. On the other hand, the observation that densities of MS granules over  $\sim 10^9$  gran/ml reduce the peroxidation of DHA suggests that highly pigmented tissue may be protected, perhaps by self-screening or because the melanin is able to function as an antioxidant through redox cycling. If it is assumed that the typical RPE cell contains from 100-200 melanosomes, and that the average cell has a volume of  $8 \times 10^{-9} \text{ cm}^3$  (assuming a cell diameter of 20 micra with a roughly cuboidal shape), then the average granule density in the RPE is at least  $1.25 \times 10^{10} \text{ gran/cm}^3$ . Therefore, the density of melanosomes may be great enough to reduce excessive photooxidation by excited pigments. The situation with lipofuscin granules, however, may be very different. Although lipofuscin granules are not nearly as photoexcitable as are the melanosomes, we have demonstrated that they have higher dark, or basal, reactivity towards fatty acids. The age-related increase of lipofuscin content in the RPE makes it likely that there is a progressive increase in oxidative stress in the RPE associated with this accumulation. Unless there is a corresponding increase in the antioxidant capacity of the cell, or sequestration of the LF granules, accumulation of LF may be a specific factor contributing to age-related retinal degenerations.

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## LIST OF ABBREVIATIONS

AOI – area of interest

DCF – 2'-7'-dichlorofluorescein (oxidized form)

DCFH – 2'-7'-dichlorofluorescein (reduced form)

DCFH-DA – diacetate ester of 2'-7'-dichlorofluorescein

DHA – docosahexanoic acid

EDTA – ethylenediaminetetraacetic acid

GSH – glutathione (reduced)

HPLC – high performance liquid chromatography

LA – linoleic acid

LF – lipofuscin granules

MLF – melanolipofuscin granules

MS – melanosomes of human (H) or bovine (B) origin

NADPH – nicotinamide adenine dinucleotide phosphate (reduced)

PUFA – polyunsaturated fatty acid

RCF – relative centrifugal force

RPE – retinal pigment epithelium

SPE – solid phase extraction

TBARS – thiobarbituric acid reactive substances

UV, UVA – ultraviolet light, A band (315 - 400 nm)

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